Molecular phylogenetic studies in the genus *Amanita*

Michael Weiß, Zhu-Liang Yang, and Franz Oberwinkler

Abstract: A group of 49 *Amanita* species that had been thoroughly examined morphologically and anatomically was analyzed by DNA sequence comparison to estimate natural groups and phylogenetic relationships within the genus. Nuclear DNA sequences coding for a part of the ribosomal large subunit were determined and evaluated using neighbor-joining with bootstrap analysis, parsimony analysis, conditional clustering, and maximum likelihood methods. Sections *Amanita*, *Caesarea*, *Vaginatae*, *Validae*, *Phalloideae*, and *Amidella* were substantially confirmed as monophyletic groups, while the monophyly of section *Lepidella* remained unclear. Branching topologies between and within sections could also partially be derived. Subgenera *Amanita* and *Lepidella* were not supported. The *Mappae* group was included in section *Validae*. Grouping hypotheses obtained by DNA analyses are discussed in relation to the distribution of morphological and anatomical characters in the studied species.

Key words: fungi, basidiomycetes phylogeny, Agaricales, *Amanita* systematics, large subunit rDNA, 28S.

Résumé : À partir d'un groupe de 49 espèces d'*Amanita* préalablement examinées morphologiquement et anatomiquement, les auteurs ont utilisé la comparaison des séquences d'ADN pour définir les groupes naturels et les relations phylogénétiques de ce genre. Les séquences de l'ADN nucléaire codant pour une partie de la grande sous-unité ribosomale ont été déterminées et évaluées en utilisant l'analyse par liaison en lacet avec le voisin (neighbor-joining with bootstrap), l'analyse en parcimonie, le regroupement conditionnel et les méthodes de ressemblance maximale. Les résultats confirment substantiellement les sections *Amanita*, *Caesarea*, *Vaginatae*, *Validae*, *Phalloideae* et *Amidella*, comme groupes monophylétiques, alors que la monophylie de la section *Lepidella* demeure obscure. On peut aussi dériver partiellement les topologies de ramification entre et à l'intérieur des sections. Il n'est pas possible de supporter les sous-genres *Amanita* et *Lepidella*. Le groupe *Mappae* est inclus dans la section *Validae*. Les auteurs discutent les hypothèses de regroupement obtenues par analyse de l'ADN en relation avec la distribution des caractères morphologiques et anatomiques aux espèces étudiées.

Mots clés : champignon, phylogénie des basidiomycètes, Agaricales, systématique des *Amanita*, grande sous-unité rADN, 28S.

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Introduction

The genus *Amanita* is one of the most familiar basidiomycetous genera, comprising about 400 species worldwide ranging from edible (e.g., *Amanita caesarea*) to deadly poisonous fungi (e.g., *Amanita phalloides*). Many species are known to be mycorrhizal fungi (see Yang 1997, and the references therein). Since Persoon introduced the genus in 1797, many mycologists have contributed to the systematics and taxonomy of the group, splitting it into smaller genera (e.g., Roze 1876; Earle 1909; Gilbert 1940) or suggesting infrageneric classification concepts (e.g., Gilbert and Kühner 1928; Konrad and Maublanc 1948; Singer 1951; Moser 1967; Garcin 1984). These systems are mainly based on morphological characters such as the presence or absence of a bulb or annulus, volva shape, form of lamellulae, and striation of the cap. An important chemical character is the spore reaction in Melzer's reagent.

A classification system accepted by many mycologists (e.g., Jenkins 1977; Hongo 1982; Ridley 1991; Tulloss et al. 1992; Fraiture 1993) was proposed by Corner and Bas (1962) and Bas (1969). Based on spore amyloidity, cap striation, and form of lamellulae, these authors separated the group into two subgenera, *Lepidella* and *Amanita*. Four sections were recognized within *Lepidella*: *Amidella*, *Validae*, *Phalloideae*, and *Lepidella;* two sections within *Amanita*: *Vaginatae*, and *Amanita.* However, some authors have expressed disagreement with this system (e.g., Singer 1975, 1986; Moser 1978; 1983, Garcin 1984).

Yang (1997) studied morphology and anatomy of about 50 *Amanita* species of different subgroups and reported a considerable uniformity in microscopic structures throughout the genus, resulting in a lack of good anatomical markers for phylogenetic reconstruction.

We have tried to expand the database for deducing hypotheses about phylogenetic relationships in the genus *Amanita* by sequencing part of the ribosomal RNA gene (rDNA). We used 587 base pairs from the 5′ end of the nu-

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clear gene coding for large subunit ribosomal RNA (LSU), a semiconservative region (Qu et al. 1988) that in the past has yielded well-resolved phylogenetic hypotheses in higher fungi at the infrageneric level (Guadet et al. 1989) as well as at higher taxonomical levels (e.g., Boekhout et al. 1995; Begerow et al. 1997). We determined DNA sequences of 49 *Amanita* species, which we evaluated by different mathematical methods.

Materials and methods

We isolated genomic DNA from *Amanita* herbarium specimens (Table 1) that had been studied previously by Yang (1997, and unpublished data). To extract DNA we followed the procedures described by Edwards et al. (1991) and Henrion et al. (1992) with modifications. A portion of 1–4 mm^2 lamella material was ground in liquid nitrogen, suspended in 500 µL extraction buffer containing SDS detergent and incubated for 1 h at 65°C. After centrifugation for 10 min at 13.793 × *g*, the supernatant was transferred to a new tube and treated with 10 units RNAse followed by a precipitation adding 1000 µL of 100% ethanol, 50 µL of 3 M sodium acetate solution and centrifuging for 15 min. The DNA pellet was then washed with 70% ethanol (v/v) and dried in a vacuum centrifuge, rehydrated in 50 µL H_2O, and stored at –20°C until use.

With the primer pair NL 1 and NL 4 (O'Donnell 1993), we performed the polymerase chain reaction (PCR; Mullis and Faloona 1987; White et al. 1990) to amplify the 5′ end region of the LSU rDNA. Reaction volume was 50 µL, with concentrations of 1.5 mM of $MgCl_2$, 200 µM of each dNTP, and 0.5 µM of each of the primers. In most cases, the following touch-down profile yielded the best results. After initial denaturation at 94°C for 3 min, 10 cycles were run with variable annealing temperatures ranging from 60°C in the first cycle to 51°C, in each cycle decreasing by 1°C, followed by 25 cycles with a constant annealing temperature of 50°C. Each of the cycles consisted of an annealing step of 0.5 min, an elongation step of 72°C for 1 min, and a denaturation step of 94°C for 0.5 min. The PCR was finished with a final elongation phase at 72°C for 7 min, after which the samples were stored at 4°C. The PCR product was purified using the QIAquick™ Kit from QIAGEN, followed by an ethanol precipitation.

We used the Perkin Elmer ABI PRISM™ dye terminator cycle sequencing kit and automatic sequencer ABI 373A to sequence the PCR product on both strands using the dideoxynucleotide chain termination method (Sanger et al. 1977).

DNA sequences were aligned with the MEGALIGN modul of the LASERGENE system (DNASTAR, Inc.), with some manual corrections. For our analyses, we chose two different species sets, set A containing 49 and set B containing 13 *Amanita* species. To both sets we added *Limacella glioderma*, a member of a genus considered to be closely related to the *Amanita* group (Moser 1967, 1978, 1983; Kühner 1980; Singer 1986). A region of 10 nucleotides ranging from position 405 to 414 of the original alignments was excluded from the analysis because of ambiguous aligning possibilities. After this, the alignment length was 587 base pairs.

The alignments were analysed by different methods. Neighbor joining (Saitou and Nei 1987) was performed with the DNAdist and NEIGHBOR modules of PHYLIP, version 3.51c (Felsenstein 1993), using Kimura two-parameter distances (Kimura 1980) as modified by Felsenstein (1993) with a transition/transversion ratio of 2.0. Bootstrap analysis (Felsenstein 1985) with 1000 alignment replicates was applied to the neighbor-joining method, using SeqBoot and CONSENSE from PHYLIP, from which also DNAml was run for maximum likelihood analysis (Felsenstein 1981) of species set B (transition/transversion ratio 2.0, default parameters of the program). Parsimony analysis was conducted using test version 4.0d56 of PAUP*, written by D.L. Swofford (1997). For set B, exact maximum parsimony analysis was carried out according to the branch-and-bound algorithm; set A was heuristically evaluated (1000 replicates of heuristic search, random addition, tree bisection–reconnection as branch-swapping algorithm, MULPARS option in effect, steepest descent not in effect). Unrooted topologies obtained by neighbor-joining, parsimony, and maximum likelihood analyses were rooted using *Limacella glioderma* as an outgroup species.

DNA sequences determined for this study were deposited in GenBank, and accession numbers are given in Table 1. Alignments can be obtained from the corresponding author.

Conditional clustering analysis

To species set A, we additionally applied the conditional clustering grouping method (Lefkovitch 1993). Given a set of objects equipped with a distance or a similarity measure, this method can detect a covering (i.e., a family of not necessarily disjoint subsets, the union of which is the entire set), which is in some sense (see below) optimal for this set of objects. Subsets contained in the covering can be considered as groups of related objects with respect to the distance data. For convenience, we will briefly review the main principles of this method here.

In the first step, candidate subsets for well-founded groups are constructed by an algorithm recursively expanding sets initialized by pairs of objects. In each round, those objects whose average distance to the current members of a subset does not exceed the maximum among the members, are included in this subset. This process is repeated until the subsets have become stable. Subsets equalling the improper subset of all objects are removed; objects not belonging to any of the candidate subsets are considered as single-object subsets (singletons).

After removal of duplicate subsets, a zero–one incidence matrix describing the membership of objects to subsets is set up. Derived from this matrix, a probability is determined for each subset giving a measure of support that this subset is contained in the optimal covering. Two strategies can then be used to chose an optimal covering: either maximizing the joint probability or alternatively minimizing the entropy of the choice. Overlapping subsets in the optimal covering can be regarded as closely connected and are finally united to form so-called musters.

For conditional clustering analysis of our molecular data, we used the CONCLUS computer program (Lefkovitch 1996) and Kimura two-parameter genetic distances nonmetrically transformed to the distances on the relative neighborhood graph (Lefkovitch 1993, pp. 206–212). These distances tend to separate distinct groups while leaving the small distances unchanged. We interpret the musters detected by conditional clustering as estimates of monophyletic groups.

Results

Analysis of species set A

Neighbor-joining analysis of set A showed distinct clusters of species, which were supported by small genetic distances between the members of the respective clusters, long intercluster distances and high bootstrap values associated with the groups (Fig. 1). Significant clusters were often consistent with sections of the *Amanita* system proposed by Yang (1997), whereas most intersectional and a large part of intrasectional relationships remained unresolved.

The following groups are well supported:

(1) section *Validae* (including *Mappae*), in our analysis represented by *Amanita citrina*, *A. citrina* var. *grisea*, *A. excelsa*, *A. flavipes*, *A. fritillaria*, and *A. pilosella*, among

Table 1. List of studied specimens.

Species	Material No.	Herbarium‡	GenBank accession No.
Amanita angustilamellata (Höhn.) Boedijn	HKAS 24158	HKAS	AF024440
Amanita avellaneosquamosa (Imai) Imai	HKAS 29500	HKAS	AF024441
Amanita brunneofuliginea Z.L. Yang	HKAS 29508*	HKAS	AF024442
Amanita caesarea (Scop.:Fr.) Pers.	C. Bas 7989	L	AF024443
Amanita ceciliae (Berk. & Br.) Bas	C. Bas 9341	L	AF024444
Amanita chepangiana Tulloss & Bhandary	HKAS 25772	HKAS	AF024445
Amanita citrina (J.C. Schaeff.) Pers.	Z.L. Yang D 33	HKAS	AF024446
Amanita citrina var. grisea Hongo	HKAS 32506	HKAS	AF024447
Amanita clarisquamosa (Imai) Imai	HKAS 29514	HKAS	AF024448
Amanita excelsa (Fr.) Bertillon	Z.L. Yang D 97	HKAS	AF024449
Amanita farinosa Schw.	RET 8-3-92-D	RET	AF024450
Amanita flavipes Imai	HKAS 32505	HKAS	AF024451
Amanita fritillaria (Berk.) Sacc.	HKAS 29511	HKAS	AF024452
Amanita frostiana (Peck) Sacc.	RET 7-25-92 E	RET	AF024453
Amanita fuliginea Hongo	HKAS 32521	HKAS	AF024454
Amanita fulva (J.C. Schaeff.) Fr.	N. Arnold 2	L	AF024455
Amanita aff. *fulva* (J.C. Schaeff.) Fr.	HKAS 29518	HKAS	AF024456
Amanita gemmata (Fr.) Bertillon	C. Bas 8942	L	AF024457
Amanita hemibapha var. *ochracea* Z.L. Yang	HKAS 29522*	HKAS	AF024458
Amanita incarnatifolia Z.L. Yang	HKAS 29519	HKAS	AF024459
Amanita japonica Bas	HMAS 59778	HMAS	AF024460
Amanita lignitincta Z.L. Yang	HKAS 29512	HKAS	AF024461
Amanita longistriata Imai	C. Bas 9040	L	AF024462
Amanita manginiana sensu Chiu	HKAS 26146	HKAS	AF024463
Amanita mira Corner & Bas	HKAS 22549	HKAS	AF024464
Amanita muscaria (L.:Fr.) Pers.	Z.L. Yang D 108	HKAS	AF024465
Amanita nivalis Grev.	R. Watling 17489†	L	AF024466
Amanita pantherina (DC.:Fr.) Krombh.	C. Bas 7474	L	AF024467
Amanita pantherina var. *lutea* Chiu	HKAS 29627	HKAS	AF024468
Amanita phalloides (Fr.) Link	Z.L. Yang D 32	HKAS	AF024469
Amanita pilosella Corner & Bas	HKAS 32517	HKAS	AF024470
Amanita pseudoporphyria Hongo	HKAS 26143	HKAS	AF024471
Amanita pseudovaginata Hongo	HKAS 29524	HKAS	AF024472
Amanita rubrovolvata Imai	HKAS 32511	HKAS	AF024473
Amanita sinensis Z.L. Yang	HKAS 25761	HKAS	AF024474
Amanita solitaria (Bull.:Fr.) Mérat	Z.L. Yang D 85	HKAS	AF024475
Amanita strobiliformis (Paul. ex Vitt.) Bertillon	M. Geesteranus 15644	L	AF024476
Amanita subfrostiana Z.L. Yang	HKAS 32513*	HKAS	AF024477
Amanita subglobosa Z.L. Yang	HKAS 12009*	HKAS	AF024478
Amanita subjunquillea var. *alba* Z.L. Yang	HKAS 24169	HKAS	AF024479
Amanita sychnopyramis f. *subannulata* Hongo	HKAS 26144	HKAS	AF024480
Amanita umbrinolutea (Secretan ex Gill.) Bataille	Z.L. Yang D 81	HKAS	AF024481
Amanita vaginata (Bull.:Fr.) Lamarck	H. A. v. d. Aa s. n.	L	AF024482
Amanita verrucosivolva Z.L. Yang	HKAS 28253*	HKAS	AF024483
*Amanita virgineoides*Bas	HKAS 18394	HKAS	AF024484
Amanita cf. *virosa* Bertillon	HKAS 27133	HKAS	AF024486
Amanita volvata (Peck) Lloyd	S. Harsch 304	RET	AF024485
Amanita aff. *volvata* (Peck) Lloyd	HKAS 26898	HKAS	AF024487
Amanita yuaniana Z.L. Yang	HKAS 29516	HKAS	AF024488
Limacella glioderma (Fr.) R. Maire	Z.L. Yang D 31	HKAS	AF024489

*Type material.

†Neotype material.

‡Herbarium acronyms: HKAS, Herbarium of Cryptogams, Kunming Institute of Botany, Academia Sinica, Kunming, Yunnan, P. R. China; HMAS, Mycological Herbarium, Institute of Microbiology, Academia Sinica, Beijing, P. R. China; L, Rijksherbarium, Leiden, The Netherlands; RET, private herbarium of R.E. Tulloss, Roosevelt, N.J.

Fig. 1. Neighbor-joining analysis of an alignment over 587 base pairs of LSU rDNA using Kimura two-parameter distances for species set A. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. Topology was rooted with *Limacella glioderma*. Numbers on branches are bootstrap values (1000 replicates, numbers rounded to next integers, values less than 70% not shown). Values in parentheses indicate the different groups obtained by conditional clustering; groups consisting of just one species are designated by (–). Tree areas corresponding to subgenus *Lepidella* are shaded. For the assignment of *Amanita strobiliformis*, *A. pseudoporphyria*, and *A. manginiana* sensu Chiu to sections, see the discussion in the text.

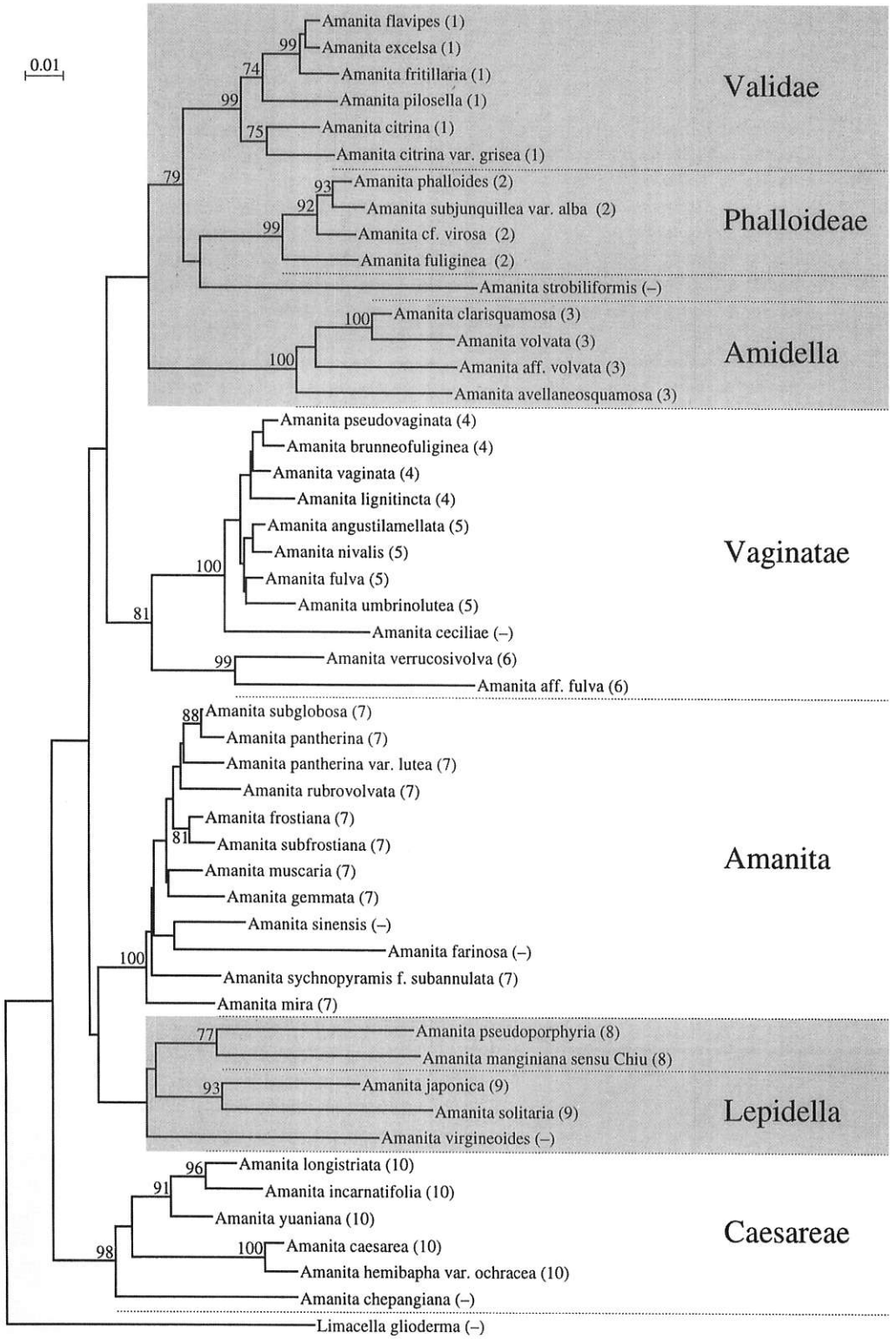


Table 2. Musters obtained by conditional clustering analysis (singletons omitted) and assignment to sections.

Muster	Section	Species
1	*Validae*	*Amanita citrina*
		Amanita citrina var. *grisea*
		Amanita excelsa
		Amanita flavipes
		Amanita fritillaria
		Amanita pilosella
2	*Phalloideae*	*Amanita fuliginea*
		Amanita phalloides
		Amanita subjunquillea var. *alba*
		Amanita cf. *virosa*
3	*Amidella*	*Amanita avellaneosquamosa*
		Amanita clarisquamosa
		Amanita volvata
		Amanita aff. *volvata*
4	*Vaginatae*	*Amanita brunneofuliginea*
		Amanita lignitincta
		Amanita pseudovaginata
		Amanita vaginata
5	*Vaginatae*	*Amanita angustilamellata*
		Amanita fulva
		Amanita nivalis
		Amanita umbrinolutea
6	*Vaginatae*	*Amanita* aff. *fulva*
		Amanita verrucosivolva
7	*Amanita*	*Amanita frostiana*
		Amanita gemmata
		Amanita mira
		Amanita muscaria
		Amanita pantherina
		Amanita pantherina var. *lutea*
		Amanita rubrovolvata
		Amanita subfrostiana
		Amanita subglobosa
		Amanita sychnopyramis f. *subannulata*
8	*	*Amanita manginiana* sensu Chiu
		Amanita pseudoporphyria
9	*Lepidella*	*Amanita japonica*
		Amanita solitaria
10	*Caesareae*	*Amanita caesarea*
		Amanita hemibapha var. *ochracea*
		Amanita incarnatifolia
		Amanita longistriata
		Amanita yuaniana

*Assignment to section uncertain.

which *A. citrina* and *A. citrina* var. *grisea*, representing the *Mappae* group, are significantly joined and placed at the base of the section;

(2) section *Phalloideae* (excluding *Mappae*): *A. fuliginea*, *A. phalloides*, *A. subjunquillea* var. *alba*, *A.* cf. *virosa*, excluding *A. manginiana* sensu Chiu and *A. pseudoporphyria*, which as a pair were placed outside the cluster and connected to *Lepidella* species present in our study;

(3) section *Amidella*: *A. avellaneosquamosa*, *A. clarisquamosa*, *A. volvata*, and *A.* aff. *volvata*;

(4) section *Vaginatae* excluding *Caesareae* (Singer 1986; Garcin 1984; Yang 1997): *A. angustilamellata*, *A. brunneofuliginea*, *A. ceciliae*, *A. fulva*, *A.* aff. *fulva*, *A. lignitincta*, *A. nivalis*, *A. pseudovaginata*, *A. umbrinolutea*, *A. vaginata*, and *A. verrucosivolva*, among which *A.* aff. *fulva* and *A. verrucosivolva* are significantly paired and placed at the base of the group;

(5) section *Amanita*: *A. farinosa*, *A. frostiana*, *A. gemmata*, *A. mira*, *A. muscaria*, *A. pantherina*, *A. pantherina* var. *lutea*, *A. rubrovolvata*, *A. sinensis*, *A. subfrostiana*, *A. subglobosa*, and *A. sychnopyramis* f. *subannulata*;

(6) section *Caesareae*: *A. caesarea*, *A. chepangiana*, *A. hemibapha* var. *ochracea*, *A. incarnatifolia*, *A. longistriata*, and *A. yuaniana*.

Section *Vaginatae* (excluding *Caesareae*) was not only detected by the different mathematical analyses we used but also marked by an insertion of 13 base pairs beginning at alignment position 53, which was present only in the members of this group.

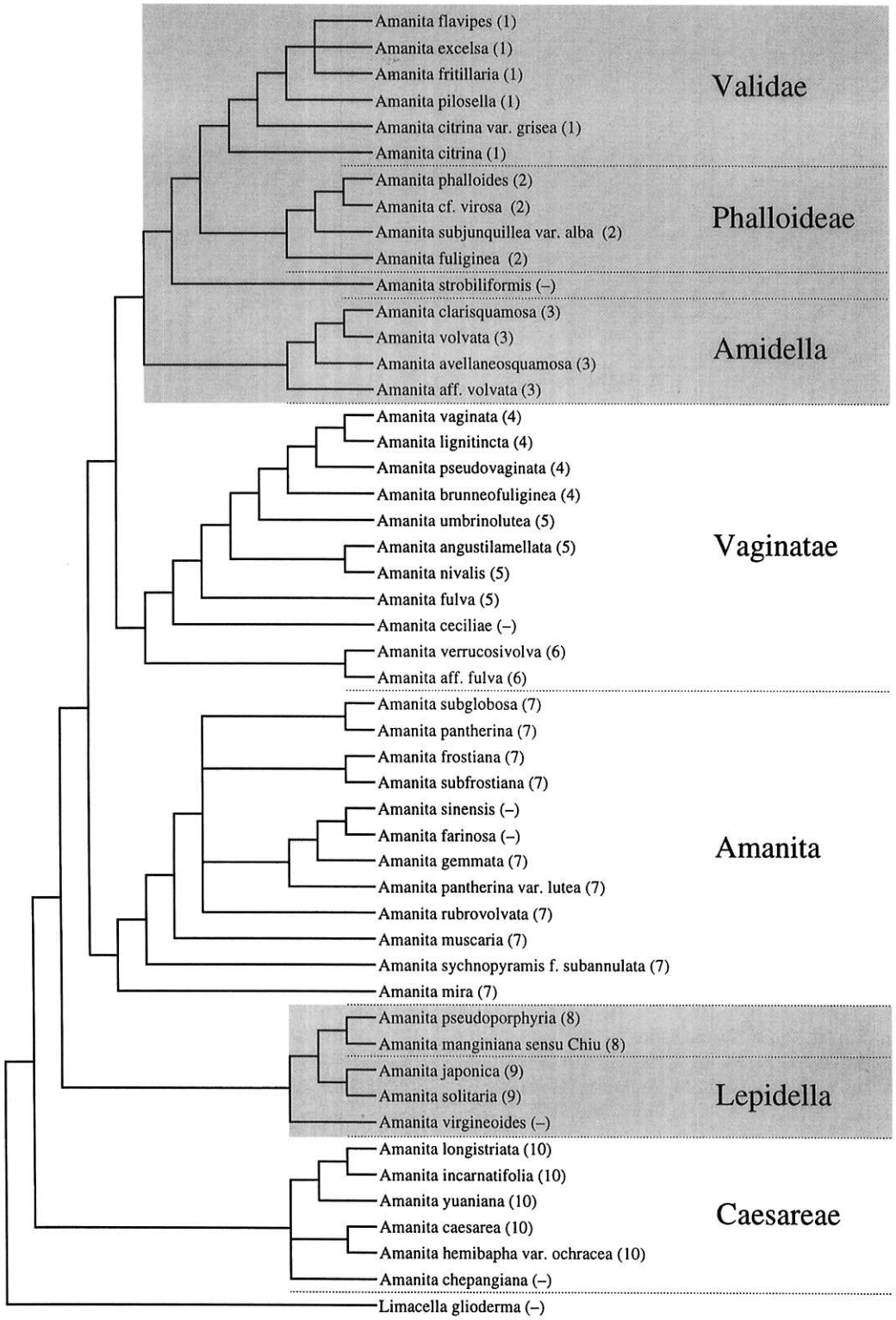
The species assigned to section *Lepidella* by Bas (1969) that were included in our study did not form a closed cluster: *Amanita japonica* and *A. solitaria* were significantly joined, clustering with *A. virgineoides* and the *Phalloideae* species pair *A. pseudoporphyria* and *A. manginiana* sensu Chiu. *Amanita strobiliformis*, usually ascribed to section *Lepidella* (e.g., Bas 1969), was connected to the *Phalloideae* group.

Our analysis did not significantly resolve the intersectional topology; yet, three of the four sections of subgenus *Lepidella* grouped together, sections *Validae* and *Phalloideae* forming sister groups linked with *Amidella* at the base.

In conditional clustering analysis, we obtained the same optimal set covering using both maximum joint probability and minimum entropy. We show the non-singleton musters in Table 2. Species groups detected by conditional clustering were consistent with those supported by neighbor joining (numbers in parentheses in Fig. 1), both methods complementing each other in resolution. There are, for example, subgroups in section *Validae* significantly supported by neighbor joining but not detected by conditional clustering analysis of full species set A and subgroups in the section *Vaginatae* detected by conditional clustering but not significantly supported by neighbor joining. Some species were not included in any of the optimal covering subsets with two or more members, forming singleton musters (labelled as (–) in Figs. 1 and 2). Since conditional clustering is not primarily designed to construct phylogenetic hypotheses but to detect groups supported by the distance data, this method gives no estimate of relationships between the detected groups or position of the single species musters.

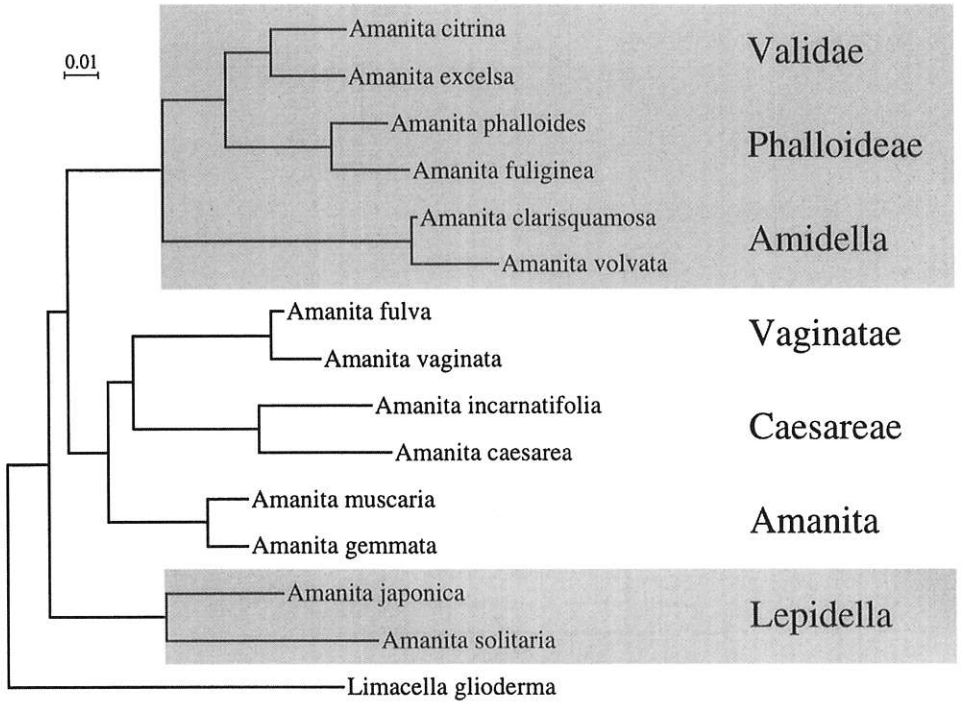
Extensive heuristic parsimony analysis of species set A yielded eight equally parsimonious best trees, each requiring 875 mutation steps. A strict consensus tree of these is shown in Fig. 2, its topology being very similar to the topology obtained by neighbor joining (Fig. 1). The same groups corresponding to sections in Yang (1997) are shown with the same exceptions mentioned above, concerning sections *Phalloideae* and *Lepidella*. Also the intersectional topology is nearly identical to that of neighbor joining, the only difference being the placement of section *Amanita:* in

Fig. 2. Strict consensus of eight most parsimonious trees obtained by heuristic parsimony analysis with 1000 replicates of an alignment over 587 base pairs of LSU rDNA for species set A. Topology was rooted with *Limacella glioderma*. Values in parentheses indicate the different groups obtained by conditional clustering; groups consisting of just one species are designated by (–). Tree areas corresponding to subgenus *Lepidella* are shaded. For the assignment of *Amanita strobiliformis*, *A. pseudoporphyria*, and *A. manginiana* sensu Chiu to sections, see the discussion in the text.



neighbor-joining analysis, sections *Amanita* and *Lepidella* are sister groups (although the dichotomy is poorly resolved), whereas in parsimony analysis, they are subsequent groups in a ladder formation (Figs. 1 and 2). Other differences are restricted to intrasectional topologies that are badly supported in neighbor-joining analysis.

Fig. 3. Maximum likelihood analysis of an alignment over 587 base pairs of LSU rDNA for species set B. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. Topology was rooted with *Limacella glioderma*. Tree areas corresponding to subgenus *Lepidella* are shaded.



Analysis of species set B

We included two species of each *Amanita* section recognized by Yang (1997) in species set B to perform maximum likelihood and an exact (branch-and-bound) parsimony analysis in addition to neighbor joining. From these analyses, we present the result of the maximum likelihood method in Fig. 3 (parsimony and neighbor-joining trees are not shown). All tree topologies obtained coincided in separating each of the sections precisely. While the placement of the inamyloid sections was variable, the relationships of the amyloid sections agreed well: *Validae* and *Phalloideae* appeared as sister groups with *Amidella* at the base, forming a closed group and separated from section *Lepidella*, which always was placed more basally.

Discussion

At the sectional level, the different types of analysis methods yielded grouping hypotheses for the most part consistent with each other and with the *Amanita* system of Corner and Bas (1962) and Bas (1969) as modified by Yang (1997). Beginning with the sections, we will now discuss our results in detail, comparing topological aspects of phylogenetic hypotheses obtained by our analyses with the distribution of morphological and other characters considered to be of systematic importance in the genus. Morphological and anatomical data are taken from Yang (1997, and unpublished data) if no other source is cited.

Section *Amanita*

A bootstrap value of 100% in neighbor-joining analysis validates the group of species belonging to section *Amanita*. Most of them are closely related by means of genetic distances and our LSU sequences provided too few nucleotide differences to resolve the inner topology of this group. This may also be the reason for different branching patterns in neighbor joining (Fig. 1) compared with parsimony analysis (Fig. 2). Consequently, it may be helpful to analyse the more variable ITS region (White et al. 1990; Bruns et al. 1991) to get a higher resolution of phylogenetic hypotheses within section *Amanita*.

With high bootstrap support in neighbor-joining analysis, *A. subglobosa* and *A. pantherina* are clustered together. Because *A. subglobosa* possesses clamps at basidial bases and *A. pantherina* is clampless, this may indicate the presence or absence of clamps as an appropriate marker in distinguishing closely related species (but compare with the role of this character in the delimitation of sections *Caesareae* and *Vaginatae* below). The linkage of *A. frostiana* with *A. subfrostiana* is well supported. Although morphologically very similar, *A. frostiana* occurs in North America and *A. subfrostiana* in East Asia. LSU sequences are different enough to justify the separation of *A. subfrostiana* from *A. frostiana.* This is also in agreement with Singer (1986), suggesting that *Amanita* species are locally restricted in their habitats.

Although not supported by a significant bootstrap value, *A. farinosa* and *A. sinensis* are paired in both neighbor-joining and parsimony analysis. A morphological trait consistent with this association is the less strongly gelatinized pileipellis of both species, in comparison with the remaining species of section *Amanita* studied. There are relatively large genetic distances separating *A. farinosa* and also *A. sinensis* from the rest of the cluster. This may indicate a higher evolution rate in this clade and also be an explanation for the fact that conditional clustering did not

include either of the two species in the group corresponding to section *Amanita* (group 7 in Fig. 1, Table 2).

Inconsistency in the placement of *A. farinosa* is also reflected by different systematic positions of this species proposed in the past. Earle (1909) described the monotypical genus *Amanitella* for it, which was accepted by Gilbert (1940). Corner and Bas (1962) assigned it to section *Amanita*. Because the cluster corresponding to section *Amanita* includes *A. farinosa* and is supported by an optimal bootstrap value in our analysis, we also think that *A. farinosa* should be retained here.

Section *Caesareae*

In this section, which was supported by a high bootstrap value of 98%, *A. caesarea* and *A. hemibapha* var. *ochracea* are grouped together in neighbor joining as well as in parsimony analysis, the pair of species being strongly supported by a bootstrap value of 100%. A morphological character correlating with this is the attachment of the volva to the stipe. In both *A. caesarea* and *A. hemibapha* var. *ochracea*, the volva is attached at the very base of the stipe whereas attachment is extended to a relatively larger area in the other species of this section.

Section *Vaginatae*

The species of section *Vaginatae* (excluding *Caesareae*) could easily be distinguished from other *Amanita* species by the presence of a characteristic insertion of 13 base pairs beginning at alignment position 53. As in section *Amanita*, most of the species of this section are too closely related as determined by genetic distances inferred from partial LSU sequences to clearly resolve the inner topology of this group. Nonetheless, two strongly supported subgroups were detected by neighbor joining, which agrees well with the grouping obtained by conditional clustering and parsimony analysis. Strikingly, one of these groups consists of *A. verrucosivolva* and *A.* aff. *fulva*, which as a pair were placed at the base of the *Vaginatae* group in both neighbor-joining and parsimony analysis. So far, we can suggest no morphological characters justifying the union of these species, but both possess a character atypical for the rest of the section. The volva on the base of the stipe of *A. verrucosivolva* is warty, while the outer surface of the volva is smooth in the other species of section *Vaginatae* studied; the pileipellis of *A.* aff. *fulva* tears radially in the margin, reminiscent of species in the genus *Inocybe*.

Amanita ceciliae could not be assigned to a section by conditional clustering and is placed at the base of the major *Vaginatae* subgroup by neighbor-joining analysis. Because inclusion of *A. ceciliae* in this group is validated by an optimal bootstrap value, and *A. ceciliae* shares the insertion of 13 base pairs mentioned above with the other species of section *Vaginatae* present in this study, there is no doubt about its membership in this section. It appears unjustified to treat *A. ceciliae* and related taxa as a separate section, as proposed by Bon (1975). The volva anatomy of *A. ceciliae* can be interpreted phylogenetically as consistent with its basal position in our analyses: volva hyphal cells are for the most part inflated in *A. ceciliae*, whereas they are more cylindrical in the remaining species of the section.

Section *Validae*

A high bootstrap support of 99% in neighbor-joining analysis was found for the group corresponding to section *Validae*. It was also identified by parsimony analysis and conditional clustering. *Amanita citrina* (= *A. mappa*) and *A. citrina* var. *grisea*, representing section *Mappae* Konr. & Maubl., which has been united into section *Phalloideae* by Corner and Bas (1962), were significantly included in section *Validae* by neighbor joining, consistent with the results of parsimony and conditional clustering analyses. Comparative anatomy of the volva and secondary metabolism also support this position: just as in section *Validae*, volva remains in the *Mappae* group are often nonmembraneous and mostly restricted to the pileus, whereas they are more membraneous and occur mostly on the stipe base in section *Phalloideae*. In contrast with members of section *Phalloideae*, *A. citrina* lacks amatoxins or phallotoxins (Wieland 1973) like the species of section *Validae*.

A high bootstrap value supports a subgroup containing *A. flavipes*, *A. excelsa*, and *A. fritillaria,* which is well separated from *A. pilosella* and the *Mappae* species *A. citrina* and *A. citrina* var. *grisea*. This grouping can also be derived from pileipellis anatomy. Terminal cells in the pileipellis are narrowly cylindrical in the *A. excelsa* subgroup, while in *A. pilosella* the terminal cells are often inflated.

Section *Phalloideae*

A *Phalloideae* cluster containing *A. phalloides*, *A. subjunquillea* var. *alba*, *A.* cf. *virosa,* and *A. fuliginea* was significantly confirmed by neighbor-joining bootstrap, concordant with the grouping hypotheses produced by parsimony and conditional clustering analyses. The pair of *A. pseudoporphyria* and *A. manginiana* sensu Chiu, which were also allocated to section *Phalloideae* (Hongo 1982; Yang 1997), is separated from this cluster in all of the analyses performed. By neighbor-joining as well as by parsimony analysis, this pair of species was found to be related, though not significantly, to *A. japonica* and *A. solitaria* of section *Lepidella*.

So far there is no morphological or anatomical data supporting this placement of *A. pseudoporphyria* and *A. manginiana* sensu Chiu, except for the fact that the two species have inconspicuous bulbs, whereas bulbs are normally well developed in members of the section *Phalloideae*. However, there might be a difference in metabolism indicating that separation of this pair of species from the *Phalloideae* group may not just be accidental. *Amanita manginiana* sensu Chiu is an edible fungus from East Asia; on the other hand, *A. phalloides*, *A. subjunquillea* var. *alba*, *A.* cf. *virosa*, and *A. fuliginea* are deadly poisonous fungi. For *A. phalloides* and *A. subjunquillea*, amatoxins and phallotoxins were shown to be the fatal agents (Wieland 1986; Kawase et al. 1992). The edibility of *A. pseudoporphyria* is still doubtful (Hongo 1957; Imazeki and Hongo 1987).

For the placement of the *Mappae* group, which was included in section *Phalloideae* by Corner and Bas (1962), see the discussion on section *Validae*.

Section *Amidella*

The species of section *Amidella* included in our study were clustered together with optimal bootstrap support in

neighbor-joining analysis and also grouped by conditional clustering and parsimony analyses. Genetic distances were big enough to resolve intrasectional relationships. Strikingly, East Asian *A. clarisquamosa* and North American *A. volvata* were directly joined by neighbor joining, associated with a bootstrap value of 100%, as well as by parsimony analysis and separated from East Asian *A. avellaneosquamosa*, which morphologically very much resembles *A. clarisquamosa*.

Our analyses confirmed Yang's opinion (1997) that *A.* aff. *volvata* should be kept inside section *Amidella* despite its inamyloid basidiospores. In the past, Bas (1969) also observed inamyloid spores in an *Amanita* species morphologically clearly assignable to section *Amidella*, which normally contains species with amyloid spores. These cases seem to exemplify local mutations of a rather conservative character in the genus *Amanita* that has been used as a marker for subgenera (e.g., Konrad and Maublanc 1948; Corner and Bas 1962; Bas 1969; Moser 1983; Garcin 1984; Singer 1986).

Section *Lepidella*

Phylogenetic hypotheses concerning the four species of section *Lepidella* included in our study are poorly resolved and should be interpreted carefully. *Amanita japonica* and *A. solitaria* were closely linked in all of our analyses, the pairing associated with a high bootstrap value in neighbor joining; *A. virgineoides* and *A. strobiliformis* could not be assigned to any cluster in conditional clustering analysis and are separated from other *Amanita* species by relatively large genetic distances. Yet placement of these two species was identical in both neighbor-joining and parsimony analysis.

Amanita virgineoides was loosely linked to the pair of *A. japonica* and *A. solitaria*, although not as closest neighbor; *A. strobiliformis* was basally connected to section *Phalloideae* inside the cluster of sections *Validae*, *Phalloideae*, and *Amidella*, which possibly form a natural group. So far, we are unaware of morphological or anatomical characters supporting the separation from *A. strobiliformis* from the other three species of section *Lepidella* present in our analyses. It is possible that section *Lepidella* consists of several heterogenous groups of species. Thus, in the future, DNA sequences of more members of the section should be analysed to develop a more meaningful hypothesis about the systematic position of its species.

Phylogenetic relationships between sections

As stated above, our analyses confirmed the division of the genus *Amanita* into subgroups, which for the most part are congruent with sections derived from comparative morphology and anatomy. The intersectional branching topology was not as well resolved in the analyses of species sets A and B. In particular, we were unable to support or falsify the division of genus *Amanita* into the subgenera *Lepidella* (shaded areas in Figs. 1–3) and *Amanita* as proposed, for example, by Konrad and Maublanc (1948), Corner and Bas (1962), Bas (1969), Moser (1983), Garcin (1984), and Singer (1986). There are, however, some aspects concordant in all our analyses that may therefore provide indications of a natural grouping of the sections.

Sections *Validae*, *Phalloideae*, *Amidella*, and *Lepidella* containing the *Amanita* species with amyloid spores and united by Corner and Bas (1962) and other authors cited above to form subgenus *Lepidella* were only partially grouped in our evaluations, leaving out section *Lepidella*, which was always isolated from the other three amyloid sections.

The union of *Validae*, *Phalloideae*, and *Amidella* was present with identical topology in all analyses, containing *Validae* and *Phalloideae* as sister groups basally linked with *Amidella*. This corresponds well with the distribution of morphological characters. As in sections *Amanita*, *Caesareae*, and *Vaginatae* containing the inamyloid *Amanita* species and in contrast to *Validae* and *Phalloideae*, the cap margin of the members of section *Amidella* is more or less striated and lamellulae are truncate. On the other hand, the species of *Phalloideae* and *Validae* have a membraneous annulus, whereas the annulus is more friable in species of section *Amidella*.

All of the evaluations performed separated sections *Caesareae* and *Vaginatae* well, which were united by Corner and Bas (1962) and Bas (1969) in their section *Vaginatae*. A combination of two morphological characters confirms this division. Species of section *Caesareae* possess an annulus as well as clamped hyphae; species of section *Vaginatae* lack an annulus and have hyphae without clamps. Tulloss (1994) reported that several *Amanita* species lack an annulus but have clamped hyphae. We have not yet examined these with molecular methods, but it would be interesting to include them in comparative analyses of DNA sequences in the future to see to what extent the combination of the characters "clamped or unclamped hyphae" and "presence or absence of annulus" is useful as a marker for natural groups in *Amanita*.

To gain a better resolution of intersectional relationships in the genus *Amanita* it would be helpful to perform molecular analyses with further *Amanita* species and larger DNA domains in the future.

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